Office of Pesticide Programs
Science Policy on

The Use of Data on Cholinesterase Inhibition for Risk Assessments of Organophosphosphorus and Carbamate Pesticides

INTERNAL DELIBERATIVE DRAFT

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Office of Pesticide Programs
US Environmental Protection Agency
Washington DC 20460

[Comments directed here to the February 28, 2000 draft as received, are those of Dr. Brian Dementi. All comments are presented in ***bold italics***, where those found also in parentheses are suggested as actual changes of wording. The bulk of the comments represent documentation, analysis and/or rationale pertaining to differences of opinion of Dr. Dementi with respect to the drafts contents. It is hoped that these comments will lead to substantial actual revisions to the Policy. B.D.]

EXECUTIVE SUMMARY

The Office of Pesticide Programs (OPP) published a policy statement on the use of data on cholinesterase inhibition (and other events associated with cholinergic effects related to nervous system function) in human health risk assessment of certain classes of pesticide chemicals for review by the FIFRA Scientific Advisory Panel (SAP) in 1997 and for public comment in 1997 and 1998 (US EPA, 1997b).The 1997 science policy document described the approaches OPP would employ in assessing the potential for human health hazard from the cholinergic effects on nervous system function following exposure to cholinesterase-inhibiting pesticides.

The 1997 policy document has been reorganized and revised, taking into consideration, as appropriate, comments offered by the public, the SAP, and other EPA offices. ***I am concerned that some of us have not had the benefit of seeing comments from other EPA offices. Also there is inadequate specific documentation cited in support of the revisions. Absence of such documentation will be noted where it occurs throughout the manuscript.*** As did the 1997 policy, this revised science policy emphasizes the weighing of all relevant evidence when selecting endpoints for the hazard assessment of anticholinesterase pesticides. This is to be accomplished by an integrative analysis after assessing all the individual lines of evidence (including all available data on cholinesterase inhibition in all compartments -- central nervous system, peripheral nervous system, red blood cells, and plasma -- as well as data on clinical signs, symptoms and other physiological or behavioral effects). Weighing of the evidence must include considerations of: adequacy of study protocols ***(including capability of detection of subtle, but meaningful neurological effects)***; quality

of data; number of studies on each endpoint; dose-dependency of responses; time course and duration of effects; and similarities or differences of responses observed in all the species, strains, and sexes tested for each duration and route of exposure evaluated.

In a weight-of-the-evidence assessment of cholinesterase-inhibiting substances, acetylcholinesterase inhibition ***(within the nervous system)*** is viewed as a key event in the mechanism of toxicity of these compounds and an important critical effect to consider in the hazard assessment. Evaluations of the cholinergic effects (i.e. physiological and behavioral changes ***Somewhere in the document there should be a section explaining what is meant by the term "behavioral changes", and not left to be misunderstood by the reader. Defining this term should be viewed as of the utmost importance, as the cholinergic nervous system is essentially ubiquitous in the CNS and fulfills a role in numerous cognitive, sensual and other phenomena. Behavioral effects should be explained as including more than clinical signs/symptoms, but as extending to effects on the higher faulties, e.g. learning and memory, that require specialized testing procedures that go beyond clinical observations to identify and characterize.*** and measures of cholinesterase inhibition in the central and peripheral nervous systems) caused by exposure to the cholinesterase-inhibiting organophosphorus and carbamate pesticides provide direct evidence for characterizing potential human health hazard. ***[***Because of likely differences in both the chemicals' and the cholinestre for the peripheral nervous system in animals and for both the peripheral and central nervous systems in humans. ***Not clear]*** Thus, information from blood cholinesterase inhibition data is considered to provide important insights into potential hazard. Red blood cell measures of acetylcholinesterase are generally preferred over plasma measures of cholinesterase activity because data on red blood cells may provide a better representation of the inhibition of the neural target enzyme, acetylcholinesterase. OPP, however, may use plasma cholinesterase inhibition data under certain circumstances, such as if red blood cell data are insufficient, of poor quality, or unavailable; if there is a lack of dose-dependency for the red blood cell acetylcholinesterase inhibition; or, if the dose responses for inhibition of plasma cholinesterase more closely approximate those for AChE inhibition in the nervous system than do the dose responses for RBC acetylcholinesterase inhibition, plasma cholinesterase inhibition may be the more prudent endpoint to use to represent the critical effect. ***Inasmuch as this text recognizes that plasma cholinesterase inhibition may more closely approximate that for AChE inhibition in the nervous system, the text should affirm an***

encumbency to make the determination in each case, as opposed to assuming that inhibition of one or the other of the blood enzyme is the more relevant. In the absence of data to make such a determination, there should be a default to the more sensitive responder of the blood enzymes as the more appropriate surrogate for absent or inadequate nervous system cholinesterase inhibition data. More will be said on this subject in subsequent passages.

It should be noted that the present policy provides guidance only on how to deal with data as they relate to the cholinergic endpoints associated with nervous system function following exposure to organophosphorus and carbamate pesticides. This scope is consistent with all earlier descriptions of Agency assessment approaches as well as that of other organizations with regard to the evaluation of cholinesterase-inhibiting substances (e.g. WHO JMPR (1998), DPR-CalEPA (1997) and other national authorities). When applying the weight-of-the-evidence approach for selecting critical effect(s) for derivation of a reference dose (RfD) or concentration (RfC), however, the entire toxicological data base on a pesticide must be evaluated (i.e., there also must be consideration of endpoints not related to the cholinergic consequences of anticholinesterase activity, for instance, liver or developmental toxicity or carcinogenicity). It is possible that, for one or more of the exposure scenarios being evaluated, the non-cholinergic effects will be identified as critical or co-critical, and they may become a more appropriate basis for deriving RfDs or RfCs.

Finally, OPP policy documents are meant to be "living documents," that is open to periodic updating and revision to reflect advances in the science. Thus, this policy, too, will be updated to incorporate important new scientific knowledge as it becomes available. For example, the routine availability of data on acetylcholinesterase activity in the peripheral nervous system may allow for refinements in the hazard assessment approach for anticholinesterase chemicals. Also, as knowledge increases about the potential roles of the different cholinesterases in the developing organism, particularly as they impact the development of the nervous system, it may allow for refinements in evaluating the potential differential sensitivity of the young versus adults. ***This statement should not be interpreted to preclude an encumbency to assess cholinesterase inhibition in adult versus young individuals <u>at this time</u> in an effort to obtain more "reliable" data in determing relative susceptability as required under FQPA.*** In fact, a substantial research effort has been, and continues to be, made to determine what roles acetyl-, butyryl- and other esterases may play in the development of the nervous system and in cell growth, proliferation and death in other tissues. OPP

encourages further discussion of the possible implications of the research findings, both for future research planning and for the Agency's regulation of cholinesterase-inhibiting pesticides. ***Please consider including the following as research objectives: 1) "Additional research is indicated to determine if in various regions (or sub-regions) of the nervous system there are loci particularly vulnerable in terms of cholinesterase inhibition and expressions of toxicity to cholinesterase inhibitors." Dementi (1997), herein after referred to as "Background Document", (p. 22); 2) "Studies suggest that repeated low level exposures to cholinesterase inhibitors can cause long term adverse efects. Additional research is recommended in order to substantiate and further quantitate this important relationship." Background Document (pp. 22-23); 3) The SAP (1997) report says that "Several Panel members noted that the importance of blood cholinesterase values in the regulation of organophosphate and carbamate pesticides has been a point of debate for decades. This conflict might be resolved by comparing the relative sensitivities of acetylcholinesterase inhibition in peripheral tissues to that noted in plasma and erythrocytes. Support for such research could be an excellent investment, since we may need to continue relying on blood cholinesterase values as the only biomarker of exposure/effect in humans. Therefore, more definitive knowledge on the utility of these markers will be essential to provide a sound scientific basis for hazard assessment and regulation." (p. 24) I should note it is affirmed in this very question posed by the SAP that uncertainty surrounds the question of the relative validity of plasma versus erythrocyte cholinesterase inhibition as biomarkers (or surrogates) of neural cholinesterase inhibition. SAP's acknowledgement of this historic uncertainty, coupled with the absence of data to resolve the issue of relative surrogacy of the two blood enzymes, is another of the many arguments why preference cannot be routinely assigned to either enzyme until the matter has been settled, either as a general principle favoring one of the enzymes, or as is more likely, in my opinion, settled as a requirement to be satisfied on a case by case basis. This is applicable with respect to the use of the blood enzymes as biomarkers of CNS as well as PNS cholinesterase inhibition. I must emphasize the importance of this in any rational approach to protect the public health, as SAP says, again, ".....since we may need to continue relying on blood cholinesterase values as the <u>only biomarker of exposure/effect in humans</u> (emphasis added)."***

LIST OF ABBREVIATIONS

Scientific Terms:

ACHE	Acetylcholinesterase
BMD	Benchmark Dose
BuChE	Butyrylcholinesterase
LOAEL	Lowest-Observed-Adverse-Effect Level
NOAEL	No-Observed-Adverse-Effect Level
PoD	Point of Departure
RBC	Red Blood Cell (or erythrocyte)
RfC	Reference Concentration
RfD	Reference Dose
UF	Uncertainty Factor

Organizational Terms:

DPR-CalEPA	Department of Pesticide Regulation-California Environmental Protection Agency
FIFRA SAP	EPA's FIFRA Scientific Advisory Panel
ILSI	International Life Sciences Institute
PMRA Canada	Pesticide Management Regulatory Agency-Canada
NRC/NAS	National Research Council-National Academy of Sciences
OPP	Office of Pesticide Programs
SAB	EPA's Science Advisory Board
SAP	EPA's FIFRA Scientific Advisory Panel
TRAC	Tolerance Reassessment Advisory Committee
WHO/FAO JMPR	World Health Organization/Food and Agricultural Organization Joint Meeting on Pesticide Residues

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The purpose of this document is to set forth the principles and procedures, including a weight-of-the-evidence approach, that will be used by OPP for the selection of appropriate endpoints for assessing potential hazards to humans exposed to anticholinesterase pesticides. In addition, this science policy document will also describe science policy approaches specific to effects related ***(to)*** cholinesterase inhibition that will be used to address inadequacies in data or lack of knowledge. The Agency's policy which addresses the potential for pre- and postnatal effects and the completeness of databases with respect to toxicity and exposure as they relate to infants and children when conducting risk assessments and making regulatory decisions regarding the setting of tolerances (residues in food) under the 1996 Food Quality Protection Act can be found in draft guidance document entitled "The Office of Pesticide Programs' Policy on Determination of the Appropriate FQPA Safety Factor(s) for Use in the Tolerance-setting Process" (US EPA, 1999) ***I examined (scanned only, have not had the opportunity to read it closely) this rather lengthy supporting document, and find no reference to cholinesterase inhibition as an end point of concern in addressing the susceptability issue under FQPA. This is rather surprising to me inasmuch as the organophosphates and carbamates have as their mode of action inhibition of cholinesterase, and that assessments of relative inhibitions of this enzyme in young versus adult individuals may well be expected to yield perhaps the most sensitive information in addressing susceptability. Indeed, the NRC (1993) expressed concerns for infants and children derived from entimates of inhibition of this enzyme. Also, to the extent the FQPA safety factor document as cited does not address relative inhibitions of this enzyme in the young versus adults, I am not certain why it is being referred to in this Policy paper on cholinesterase. On the other hand, there should be no reticence to briefly cite the document in this cholinesterase Policy in the appropriate sections where cholinesterase inhibition in young versus adult animals would be necessary in risk assessment. In any case, this cholinesterase Policy, under its own recognizance, should affirm***

the importance of taking cholinesterase data in both young and adult animals (e.g. in reproduction, developmental toxicity, developmental neurotoxicity, possibly acute studies, etc) to address the susceptability issue under FQPA. Again, where cholinesterase inhibition is concerned, such important information as may be found in another document should be at least briefly cited here.

Regulatory decision making in EPA is described as consisting of two major steps--risk assessment and risk management[s]1 to occur in individuals or populations, while risk management weighs risk reduction alternatives and integrates the risk assessment with social, economic, and other factors, as appropriate. The Agency uses the paradigm put forward by the National Research Council of the National Academy of Sciences in 1983 and modified in 1994 (NRC/NAS, 1983; 1994) that defines and organizes risk assessment into four phases: hazard identification, dose-response assessment, exposure assessment, and risk characterization. Risk assessment for noncancer effects including those addressed in this policy is generally based on identifying a no-observed-adverse-effect[t]2, which is usually determined from laboratory animal studies for use as a Point of Departure (PoD) when deriving a reference dose (RfD) or reference concentration (RfC) . The PoD is divided by one or more uncertainty factors (UF). These UFs (typically 3- or 10-fold in magnitude) reflect uncertainties inherent in the extrapolation from laboratory animal species to humans (interspecies UF), in the variations in sensitivity among members of the human population (intraspecies UF), for the use of subchronic rather than chronic data (subchronic to chronic UF), the use of a lowest-observed-adverse-effect level (LOAEL) rather than a NOAEL (LOAEL to NOAEL UF), and the comprehensiveness and quality of the database available, i.e., whether or not all potential endpoints of concern are identified and evaluated in acceptable studies (database UF). A modifying factor (MF) may be used to address scientific uncertainties in the principal study used for RfD/C derivation which are not explicitly addressed by the other standard UFs.

The result of dividing a PoD by the appropriate uncertainty factors and/or a modifying factor is a reference dose (RfD) for oral or dermal exposures–or reference concentration (RfC) for inhalation exposure(s). The RfD or RfC is defined as an estimate, within an order of magnitude, of exposure assumed to be without appreciable risk for adverse noncancer health effects. In the risk

characterization step, the RfD and RfC values are compared to potential or known exposure levels. Risk characterization also fully describes the nature and extent of the risks posed, and how well the data support the conclusions, including a discussion of the limitations and uncertainties involved. Sometimes, because of these limitations and uncertainties, further data may be collected to reduce the uncertainties and refine the risk assessment.

The Agency has acknowledged that the historical approach to defining a NOAEL and calculating RFDs and RfCs has limitations (see USEPA, 1994; 1995; 1996). In response, the Agency has developed guidance on an alternative method–the Benchmark Dose (BMD) Approach (USEPA, 1996). The BMD is defined as the statistical lower confidence limit on the dose producing a predetermined level of change in response compared with the background response. A BMD is derived by fitting a mathematical model to the dose.[3] The Agency is still gaining experience with BMD analyses and has not yet formally finalized standard operating procedures. OPP, however, will use the BMD approach for derivation of RfDs and RfCs to the extent possible.

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Acetylcholine is a neurotransmitter which enables chemical communication to occur between a nerve cell and a target cell. This target cell may be another nerve cell, muscle fiber or gland. Upon stimulation, the nerve cell releases acetylcholine into the synapse (or space) between the two cells. This released acetylcholine binds to receptors on a target cell, thereby passing the signal on to that nerve cell, muscle or gland. The end result of the stimulation of cholinergic pathway(s) includes the contraction of smooth (*e.g.*, in the gastrointestinal tract) or skeletal muscle, changes in heart rate or glandular secretion (*e.g.*, sweat glands). Cholinergic pathways innervate virtually every organ in the body. ***The role of the cholinergic system within the CNS, in addition to the fact that many central neurologic phenomena are consequently involved, should also be acknowledged in this paragraph.***

There are two major divisions of the nervous system, both of which contain cholinergic pathways that may be affected by cholinesterase-inhibiting chemicals:

ÿ the peripheral nervous system, consisting of neuromuscular junctions in skeletal muscle, and tissues of the autonomic nervous system, consisting of ganglia of the sympathetic and

parasympathetic nervous systems, smooth muscles, cardiac muscle, and glands; and

- ÿ the central nervous system, consisting of brain and spinal cord.

The distribution of cholinergic receptors in the central nervous system and the peripheral nervous system is not uniform (Brimijoin, 1992). For example, certain brain regions of the mature organism are rich in cholinergic neurons (*e.g.*, the striatum), while other regions have few, if any, of such neurons (e.g. the hippocampus, cerebral cortex and the olfactory bulbs). ***In reading this statement, one might get the impression that the cholinergic system plays little or no role in the latter three important areas of the brain. Alternatively, consider the following information. There are many references attesting to the essential ubiquity of the cholinergic system in the CNS. In the Background Document, it is noted that: "Muscarinic receptors are located in smooth muscle and the heart, at some autonomic ganglia and in many brain regions, most notably the striatum, hippocampus and cerebral cortex." (p. 27) There are many other references, e.g. Mesulam (1995) [Mesulam, M.M. "Structure and function of cholinergic pathways in the cerebral cortex, limbic system, basal ganglia, and thalamus of the human brain" in Psychopharmacology: The Fourth Generation of Progress, F.E. Bloom and D.J. Kupfer, eds., Raven Press, N.Y. 1995, Chapt. 12, pp. 135-146] says: "There are eight major cholinergic cell groups that project to other central nervous system structures. Most of these cholinergic cell groups do not respect traditional nuclear boundaries, and their constituent cholinergic cells are intermixed with other noncholinergic neurons. We have therefore introduced the Ch1-8 nomenclature in order to classify the cholinergic neurons within these eight cell groups." "Tracer experiments in a number of animal species have shown that Ch1 and Ch2 provide the major cholinergic innervation of the hippocampal complex, Ch3 for the olfactory bulb, Ch4 for the cerebral cortex and amygdala, Ch5 and Ch6 for the thalamus, Ch7 for the interpeduncular nucleus, and Ch8 for the superior colliculus. There are also lesser connections from Ch1-Ch4 and Ch8 to the thalamus and from Ch5-Ch6 to the cerebral cortex" (pp. 135-136); According to Russell and Overstreet (1987) [Russell, R.W. and Overstreet, D.H. "Mechanisms underlying sensitivity to organophosphorus anticholinesterase compounds", Progress in Neurobiology, 28, 97-129; G.A.Kerkut and J.W. Phillis, eds., Pergamon Journals Ltd. Oxford, England 1987]: "In the classical literature of ACh, three major morphological systems are mentioned: the***

septohippocampal pathway, with cell bodies in the medial septal nucleus and terminals in the hippocampus; the cerebral cortical system, composed of intrinsic interneurons and extrinsic neurons arising from cell bodies in the basal forebrain nuclei including the nucleus basalis; and, the striatal system, composed primarily of intrinsic interneurons." (p. 102); Palacios et al (1991) [Palacios, J.M., Boddeke, H.W.G.M. and Pombo-Villar, E. "Cholinergic neuropharmacology: an update", Acta Psychiatr Scand 1991: Suppl 366, 27-33] say: "When the characteristics of MChR (muscarinic cholinergic receptors) in different organs and tissues are examined, the brain is found to be one of the organs with the highest density of MChR. The majority of these receptors belong to the M1 subtype. M1 receptors are particularly enriched in the neocortex and the hippocampus, 2 brain areas known to play an important role in learning and memory processes." (p. 30) There are two major types of cholinergic receptors – muscarinic and nicotinic -- and there are several subtypes of each. These receptor types are also differentially distributed in different regions of the central and peripheral nervous systems, thus contributing to the complexity of effects that may occur.

Acetylcholinesterase (AChE) is found in cholinergic neurons, in the vicinity of synapses, and in other, non-neural tissues. It is highly concentrated at the neuromuscular and other neuroeffector junctions. It is the enzyme that breaks down acetylcholine and terminates its action in the synapses between neurons and between neurons and muscle fibers or glands. Inhibition of AChE leads to an accumulation of acetylcholine and a prolongation of the action of acetylcholine at the nerve-nerve, nerve-muscle or nerve-gland interface. Peripherally, the accumulation of acetylcholine can result in cholinergic responses such as smooth muscle contractions (*e.g.*, abdominal cramps), glandular secretions (*e.g.*, sweating), skeletal muscle twitching, and, at higher concentrations, flaccid paralysis. In addition, there may be centrally-mediated effects on learning, memory and other behavioral parameters. Thus, the inhibition of AChE potentially results in a broad range of adverse effects, having an impact on most bodily functions, and depending on the dose ***(and its duration)***, these effects can be serious or fatal. ***The interpretation of "serious" in this statement should be explained as embracing more than the litany of classical cholinergic signs/symptoms, but to include the more <u>subtle</u>, perhaps difficult to characterize, cognitive effects (e.g. learning and memory) that most people would find disturbing, particularly if affecting academic performance in their school children. OPP needs to rise to the occasion of addressing this concern.***

Effects caused by AChE inhibition may be a result of action on ***(the cholinergic system within)*** the central nervous system ***(and or)*** the peripheral nervous system. Access of chemicals to the central nervous system and the peripheral nervous system may be different because of differences in pharmacokinetic properties of these two compartments (*e.g.*, differences in absorption, distribution, metabolism, elimination). The pattern of effects seen may also depend upon factors such as the pharmacodynamic characteristics (*i.e.*, binding potency, rate of reversal) of the cholinesterase-inhibiting chemical and the molecular form of cholinesterase with which it is interacting (e.g. see Scarsella, et al., 1979). ***When speaking of access of chemicals to the nervous system, mention should be made of the "blood-brain barrier", a regulatory interface of considerable importance. It is very important to explain that the blood-brain barrier may be poorly developed in young individuals, and is all the more reason for taking cholinesterase data on young individuals as suggested above. In support of this, the National Academy of Sciences', National Research Council (1993) report "Pesticides in the Diets of Infants and Children" says: "There is speculation that neonates and infants may be more susceptible to chemically induced neurotoxicity, in part because of the immaturity of their blood-brain barrier. Watanabe et al (1990) point out that the central nervous system in developing individuals is potentially vulnerable to chemicals for a protracted period because the central nervous system requires longer than most other organ symptoms for cellular differentiation, growth, and functional organization. Therefore, any increase in accessibility to cytotoxic agents because of delayed maturation of the blood-brain barrier could have serious consequences." (p.89) This being true, a concern exists that cholinesterase inhibiting pesticides might more readily enter the CNS of the young, but in endeavoring to regulate these chemicals properly, how would we know, lest the proper assays are performed that might reveal the differential susceptability? The importance of addressing this issue of susceptability, as identified by assessments of this most fundamental end point of cholinesterase activity in the young, is further attested to in NRC (1993): "Assessment of the effects of pesticides on the developing human nervous system is difficult because the methodology for such assessment is complex and poorly delineated. Development of the CNS is characterized by exacting architectural complexity and localization of function occurring over a prolonged period postnatally. The effects of altered neurological development may be measured either as anatomic or behavioral and cognitive outcomes." (p. 108)***

Butyrylcholinesterase (BuChE) is similar in structure to AChE, but it is encoded by a separate gene. BuChE, ***(the plasma form of)*** which is synthesized primarily in the liver, is generally distinguished from AChE by BuChE's slower rate of hydrolysis of acetylcholine, by function and by localization using histochemical techniques after subjecting the experimental model to inhibitors which selectively block the activity of one but not the other enzyme (Taylor and Radic, 1994). Furthermore, the binding affinity of anticholinesterase chemicals for each enzyme can differ among these substances (Silver, 1974; Taylor and Radic, 1994). ***In a balanced comparison between the two cholinesterases, more needs to be said here of the similarities, e.g. as explained in the Background Document, both exist in an analogous set of six molecular forms (G1, G2, G4, A4, A8 and A16), both hydrolyze choline esters, AChE having greater specificity for acetylcholine and both are inhibited by cholinesterase inhibiting xenobiotics. Although the two enzymes differ in specificity for binding acetylcholine and other choline esters, the difference in specificy toward cholinesterase inhibiting xenobiotics may be less, since binding is less circumscribed for such agents than for the substrate (choline esters), and furthermore, such differences may be dwarfed by the host of unpredictable and poorly understoo <u>in vivo</u> parameters that may overide in determining relevance to neural cholinesterase inhibition. One must obtain the data.*** Both enzymes are present during development of the nervous system, with the ratios of one to the other changing substantially over time and with location (Hoffman, et al., 1996). While no biological function has been shown definitively for BuChE in the

developing or mature nervous system, the function of BuChE present in the plasma appears to be the hydrolysis and inactivation of ingested esters from plant sources (e.g. cocaine and related synthetic local anesthetics (Lefkowitz, et al.,1996) ***Reference not found in Bibliography*** and neuromuscular blocking agents such as succinylcholine (Taylor, 1996b). ***Consider the following alternative language for the last sentence: (The physiological or biochemical function of BuChE is unknown. It is recognized, however, that BuChE present in the plasma will catalyze the hydrolysis and inactivation of ingested esters from plant sources (e.g. cocaine and related synthetic local anesthetics) (Lefkowitz, et al., 1996) and neuromuscular blocking agents such as succinylcholine (Taylor, 1996b). Likewise, there is no known physiological or biochemical function for erythrocyte AChE (Brimijoin, 1992)(p. 23); Dementi (1997) (p. 8).)***

As discussed later, the blood cholinesterase enzymes are regarded, as a matter of policy, as

surrogate measures of neuronal cholinesterase activity. Of the two common blood elements measured, red blood cells (RBC) contain AChE exclusively, while the ratio of AChE to BuChE in plasma varies widely among humans, dogs, and rats, the species in which these measures are most typically made for risk assessment and regulatory purposes. While human plasma is overwhelmingly BuChE, the plasma of dogs and rats contains both AChE and BuChE. ***Reference ?***

The question of whether, and, if so, how, BuChE plays a role in the development and/or functioning of the nervous system still awaits resolution. Work currently is underway to determine whether, and, if so, how, butyrylcholinesterase plays a role in nervous system morphogenesis (development) and function, and whether, and if so, how, butyryl- and/or acetylcholinesterase and other esterases play a more general role in cell growth and death, including in carcinogenesis. In addition, the dose response relationships attendant to acetylcholinesterase's function(s) in the development of the nervous system must be developed and compared with those of the endpoints currently used in the evaluation of nervous system function. OPP is preparing a brief summary of the available literature on the role of the cholinesterases (and, perhaps, other esterases) in these areas. ***[***OPP also is preparing a series of questions for consideration before modifications and revisions to the present policy can be justified.***]*** ***Just exactly what this latter statement means is unclear.*** This effort is occurring separately from the revision of this policy document to serve as a starting point for discussion for addressing these important issues.

Cholinesterase inhibition and the cholinergic effects (*i.e.,* the physiological or behavioral changes) caused by organophosphorus and carbamate pesticides have long been endpoints that OPP has used in assessing potential human health hazards. For well over a decade, OPP has regarded data showing cholinesterase inhibition in brain, RBC, or plasma, and data on physiological or behavioral changes as critical effects (*i.e.*, effects that should be considered for use in the derivation of an RfD or RfC). OPP has used statistical significance, rather than a fixed percentage of response from baseline, as the primary, but not exclusive, determinant of toxicological and biological significance in selecting Points of Departure (e.g. NOAELs or LOAELs or Benchmark Doses). The use of uncertainty factors and the use of statistical significance are consistent with Agency practice for all

non-cancer, systemic toxicity endpoints.

OPP's Reference Dose Tracking Report (US EPA, 1997a) lists chronic Reference Doses for over 50 chemicals based in whole, or in part, on cholinesterase inhibition. There are, however, many more than 50 risk assessments that make use of this endpoint in acute and chronic dietary exposure/risk assessments and in other, non-dietary scenarios representing both short-term and intermediate-term exposure(s).

Prior to 1997, one internal Agency colloquium (US EPA, 1988) and two public Science Advisory Board (SAB)/Scientific Advisory Panel (SAP) meetings (SAB/SAP, 1990; 1993) considered Agency draft guidance on the use of cholinesterase data in risk assessment. An additional SAP/SAB review in 1992 of a proposed reference dose for aldicarb also addressed the issue of cholinesterase inhibition as an endpoint in risk assessment (SAB/SAP, 1992). Each of these reviews yielded somewhat different perspectives and recommendations, based in part on somewhat differing proposed policies, but primarily on differing points of view of each peer review group. The area of greatest divergence among these reports and in their recommendations involved the interpretation and use of blood measures of cholinesterase inhibition, particularly in plasma, for deriving reference doses. Some reviewers and panels placed less (or no) reliance on plasma measures of cholinesterase inhibition and/or less reliance on red blood cell measures of AChE inhibition as a critical effect than OPP traditionally has placed on each. The Agency has never finalized guidance on this topic.

In 1997, OPP published its own policy statement on the use of data on cholinesterase inhibition for risk assessments, accompanied by case studies illustrating the application of this policy and a review of pertinent literature on cholinesterase inhibition prepared by OPP staff for public comment and SAP review (US EPA, 1997b; Dementi, 1997). ***As indicated above, prior to the June 1997 SAP meeting pertaining to this policy, certain very important questions as to the toxicology of cholinesterase inhibition and just how to employ cholinesterase inhibition itself in both the assessment of adversity and in the regulatory setting had been left standing with much uncertainty. The lingering uncertainties prompted a decision within the Agency to address these issues more definitively through a review of the literature, and the drafting of a policy statement, accordingly. The 1997 Policy Statement, that was endorsed by that 1997 SAP settled much that was previously unresolved. Thus the 1997 SAP was not just one of so many panels to comment***

on the subject, rather it was the defining effort in establishing a policy. In other words, the 1997 SAP, which took into consideration the earlier works cited, was the defining activity among those cited in this section. Yet, I perceive it is being marginalized in this February 28, 2000 draft revised Policy, without justification nor opportunity for comment by the very members of the 1997 SAP panel that endorsed the Policy. Clearly, if external peer review means anything, insofar as it may be possible, this altered Policy must be submitted for comment by those SAP Panelists who were involved In 1998, as part of the OPP review process for science policy issues agreed upon in conjunction with the Tolerance Reassessment Advisory Committee (TRAC), OPP again made ***(both)*** the 1997 policy paper ***(and the attendant supporting Background Document, Dementi (1997), Parts A and B)*** available for broader public comment (US EPA, 1998b). ***This November 5, 1998 offering for public comment pertained in part to the Food Quality Protection Act (FQPA). It was intended to address the use of cholinesterase data in satisfying requirements for both completeness of data and its reliability as established under FQPA, where decisions regarding the retention or removal of the FQPA imposed 10X safety factor for the protection of infants and children is concerned. Since the FQPA issue was a focus of the 1998 public comment offering, more needs to be said, if only briefly, characterizing the comments received.***

The 1997 OPP policy statement described a weight-of-the-evidence approach for use when evaluating the data on cholinesterase inhibition and its consequent potential adverse cholinergic effects. The 1997 policy paper also proposed that the differing opinions with respect to ***(the use of)*** blood measures ***(of cholinesterase inhibition as surrogates for neural cholinesterase inhibition)*** could be reduced or resolved by the collection of peripheral nervous system tissue measurements of AChE inhibition in animal studies which might serve instead of the blood measures as critical effects for use in hazard assessment. The SAP favorably received this proposal (SAP, 1997). Briefly, the SAP stated that:

*... the weight of evidence {approach} is indeed reasonable and justified on the basis of the available scientific data so long as these data are derived from rigorous experiments with standardized methods and proper controls. In particular, this approach allows flexibility to weight heavily inhibition in non-target tissue when the overall toxicologic context suggests that other approAChEs pose danger of serious risk from overexposure...**(p. 20)** There was unanimous support for the notion that, under SOME circumstances, measurement of SOME blood-borne cholinesterases would be*

*appropriate to consider in establishing RfDs for anticholinesterases...**(p. 21)** . . . measured inhibition of cholinesterase activities in any of the blood fractions is best regarded as an imperfect mirror of enzyme inhibition in the true target tissues**: . . . (p. 21)***

The 1997 SAP further concluded that the use of blood measures "is readily justified if the discrepancy between blood cholinesterase and functional endpoints is not too great" and recommended that data on AChE inhibition in the peripheral nervous system be collected. ***It should be acknowledged here that the 1997 SAP affirmed the supporting literature review (i.e., the Background Document) in its completeness; a significant work. Indeed , when SAP was asked the question: "Does the review include the major concepts and citations from the literature and present an overall <u>objective</u> (emphasis added) analysis consistent with the proposed policy?, "The Panel gave a strongly positive answer." (p. 19) Hence, efforts to remove or marginalize the Background Document do so in the face of a remarkable endorsement by outside experts convened for the very purpose of evaluating the information. In particular, it is important to note that in the many places within the SAP final report wherein comments were rendered on the use of blood enzyme cholinesterase inhibition, rarely was distinction made by the Panel as to the relative importance of erythrocyte and plasma cholinesterase inhibition as surrogates for absent or inadequate neural cholinesterase data. This is consistent with the concept, as presented in the Background Document and buttressed by SAP's endorsement, that: "As to the relevance of plasma cholinesterase and erythrocyte acetylcholinesterase inhibition to nervous system acetylcholinersterase inhibition, it is self evident that the numerous <u>in vivo</u> biochemical and physiological parameters may weigh far more heavily in determining whether inhibition of one blood enzyme or the other is the better correlate. Again this must be determined on a compound by compound basis, and to be definitive would require an extensive data base on a given inhibitor." (p. 18) In other words, SAP (1997) affirmed that both enzymes are equally relevant until established otherwise. Also from the Background Document: "In the absence of fully documented evidence to the contrary for a given organophosphate or carbamate, equal importance should be ascribed to plasma and erythrocyte cholinesterase inhibition as surrogates for inhibition of the nervous system enzyme and of toxicity. This position is rationalized after molecular considerations (presented in the Discussion section) as well as by certain actual case studies in which plasma cholinesterase was either a very excellent or, with reference to erythrocyte cholinesterase, the preferred surrogate for inhibition of the nervous system enzyme." (p. 80)***

Furthermore, there are several other important topics that were under developed in the Background Document, and endorsed by the SAP that do not find expression in this Policy Statement. For example, the concept of "tolerance", an import subject explaining how animals appearing very normal clinically following exposures to cholinesterase inhibitors, may have extensive changes in neurochemistry and respond anything but normally under the influence various insults or specific testing procedures.

OPP subsequently asked the International Life Sciences Institute (ILSI)/Risk Science Institute to

convene a workgroup to help further define the feasibility and details for collecting these data This workgroup's report concluded that it was currently feasible to measure AChE inhibition in the peripheral nervous system (Mileson *et. al.,* 1999). The ILSI workgroup further concluded, "Methods and techniques currently available are adequate to characterize the AChE activity in the peripheral nervous system, but additional studies would help to improve these methods."

This Chapter explains the science policy decisions and rationale for evaluating the various cholinergic effects on nervous system function caused by anticholinesterase pesticides. This rationale forms the basis of the weight-of-the-evidence approach described later in Chapter 4. This Chapter is organized around conclusions followed by a rationale addressing three key types of endpoints generally assessed currently for cholinesterase-inhibiting pesticides: 1) evaluations of physiological and behavioral effects ***As suggested earlier, "behavioral effects" is terminology requiring characterization somewhere in this Policy statement, such as in a footnote on this page analogous to that provided for signs and symptoms.*** ; 2) measures of acetylcholinesterase inhibition in the neural tissues (*i.e.*, brain and peripheral nervous system);and, 3) measures of cholinesterase inhibition in the blood (*i.e.*, red blood cells and plasma).

Conclusions:

- [ss]5 in humans and behavioral or physiological effects in animals provide the most direct evidence of the potential adverse consequences of human exposure to anticholinesterase pesticides.

- [ss]6 can cover a broader range than those that can be observed in animal studies, including psychological complaints, cognitive complaints, and other subjective effects. ***Effects may also be reflected in performance on psychometric testing, e.g. learning and mamory, behavior, etc. Please refer to the following passages in the Background Document for identification of some of the many end points that may be involved: Wolthuis et al (1995) (p. 33); Karczmar (1984) (p. 60); Savage et al (1988) (p. 67)*** Human studies following either deliberate or inadvertent exposure,

nevertheless, are currently quite limited in the scope of the evaluations made and scale of the measurements used. Also, the generally small numbers of subjects may limit the power of the study to detect effects of concern.

- Evaluation of physiological and behavioral changes (*i.e.*, functional data) ***"Functional data" is no more effective than "behavioral changes" in conveying to the reader the character of the end points under consideration. This term "functional" is used very liberally beyond this point in the text, and appears to refer to any end point or finding other than cholinesterase inhibition itself. Again, "behavioral" requires specific characterization, i.e. delineation of end points covered.*** in animal studies also are limited in terms of the scope of effects assessed and the measurements employed. It is possible that one or more effects of concern may be occurring but measures ***(procedures)*** for their evaluation ***(either lacked sensitivity or)*** were not incorporated in the study design. Thus, we may be left with the situation of a false negative. ***(Under such circumstances, more reliance must be placed on cholinesterase inhibition as a fundamental end point for agents designed to inhibit this enzyme.)***

- Because of the limited range of measures of behavioral and physiological effects evaluated historically, functional data obtained from human and animal studies should not be relied on solely, to the exclusion of other kinds of pertinent information, when weighing the evidence for selection of the critical effect(s) that will be used as the basis of the RfD or RfC.

Rationale:

Many of the adverse acute and longer-term effects of anticholinesterase organophosphorus pesticides that have been observed in humans were described by Morgan (1989) and updated by Reigart and Roberts (1999):

Most commonly reported in humans are headache, nausea, and dizziness. Anxiety and restlessness are prominent. Worsening may result in muscle twitching, weakness, tremor, incoordination, vomiting, abdominal cramps, diarrhea. Often prominent are sweating, salivation, tearing, rhinorrhea, and bronchorrhea. Blurred and/or dark vision, and excessive contraction of the pupil

of the eye (miosis) may also be seen. Tightness in the chest, wheezing and productive cough may progress to frank pulmonary edema. Bradycardia may progress to sinus arrest, or tachycardia and hypertension. Confusion, bizarre behavior, and toxic psychosis may occur. In severe poisonings, toxic myocardiopathy, unconsciousness, incontinence, convulsions, respiratory depression and death may be seen. Repeated absorption, but not enough to cause acute poisoning may result in persistent anorexia, weakness, and malaise.

As noted, many of the effects described above may be seen after acute exposures to anticholinesterase pesticides. There also are ***(published works in animal models and human)*** case reports describing long(er)-term effects following acute exposures, ***(Dementi (1997).) The acute clinical signs/symptoms presented above are essentially those that follow high dose exposures to cholinesterase inhibitors. Of equal or perhaps greater importance where the broader public health is concerned might be effects on behavioral end points of acute low dose (i.e. sub-clinical in terms of cholinergic signs) exposures to such agents. As presented in the Background Document there are published works indicating effects on complex behaviors following such <u>single low dose</u> administration of a number of cholinesterase inhibitors [Wolthuis and Vanwersch (1984); Wolthuis et al (1995); Kurtz (1976); Kurtz (1977); Burchfiel et al (1976); Pope et al (1992); Nieminen et al (1990); Weinstock et al (1994); Bowers et al (1964) (a human study in which clinical signs such as pupillary constriction, bronchoconstriction, hypermotility of the lower bowel, muscle fasciculation were not seen. Nausea and vomiting were the only signs.)]. A quotation from one of these publications, Wolthuis et al (1995), serves to illustrate the concern being raised here: "The objective of this study is to contribute to an assessment of the risk that exposure to ChE inhibitors may cause subtle disruptions of 'higher' CNS functions that may go unnoticed because physical signs are absent. Particularly suspect are compounds such as physostigmine and soman, which easily penetrate the blood-brain barrier and may act on the CNS <u>at low dose levels</u> (emphasis added). Extrapolated to man, it may mean that such subtle disruptions of CNS functions affect decision making, logic, memory, etc., which are all vital for complex operations." (p. 444) This is the concern, and the publications cited appear in recognized journals. Clearly more work needs to be done in this area, and this cholinesterase Policy paper should identify this as a research need. Nonetheless, the cited studies do appear in***

the literature, and should be acknowledged in the Policy under section 3: "<u>Identification of the Toxicological Endpoints for Assessment of Cholinesterase inhibitors</u>".

No credible information exists describing effects following long(er)-term, low-level exposures. ***Toward presenting a balanced assessment of the subject of possible longer term effects resulting from low level exposures, more needs to be said than simply*** "No credible information exists describing effects following long(er)-term, low-level exposures." ***Principle sections in the Background Document involved presentation and discussion of published works on the possible long term (subchronic; chronic) effects resulting from longer term low-level (even subclinical) exposures to cholinesterase inhibitors [Desi et al (1974); Desi et al (1976); Burchfiel et al (1976); Geller et al (1985); Bushnell et al (1991); Bushnell et al (1994); Padilla et al (1993); Nagymajtenyl et al (1988); Stephens et al (1995) (epidemology study)]. Also, the concerns posed by the development of "tolerance" and adaptability in general of the central nervous system need to be mentioned. The report of the joint SAB/SAP (1993) meeting on cholinesterase concluded that: "Repeated exposures to cholinesterase-inhibiting pesticides could, therefore, fail to produce the typical overt signs of acute toxicity in animals or persons exhibiting extensive changes in neurochemistry." (p. 9) Illustrating this very point where repeated low-dose (absence of any discernible symptoms) organophosphate exposures result in asymptomatic animals that exhibit <u>abnormal</u> behavioral responses when challenged pharmacologically, is that of Annau (1992). Again, these sections were part of the Background Document as presented to the SAP(1997), where it received a very favorable review by the panel. In the interest of <u>transparency</u> (a topic of considerable interest itself where Agency deliberations are concerned) and completeness of the historical record, one might ask, <u>in what forum were decisions rendered that the published evidence presented to the SAP (1997) is not credible, being mindful such decisions compromise that which was accomplished at the 1997 SAP meeting?</u>***

The Department of Veterans Affairs (1998) [Annual Report to Congress. Federally Sponsored Research on Gulf Was Veterans' Illnesses for 1998, dated June 1999] in its Annual Report to Congress on Persian Gulf Veterans Illnesses examined a number of subjects relevant to the question of whether veterans experience adverse health effects resulting from the Gulf experiences. This annual report provides summaries of recently published research findings and

a general overview of Gulf War veterans'illnesses. It is evident in this work that certainly two issues of concern related to possible effects of exposures to cholinesterase inhibitors are those of long term adverse effects of low-level (sub-clinical) exposures to organophosphates, particularly chemical warfare agents, but insecticides and other ops are included as being of interest. Also of expressed concern are possible effects of pyridostigmine bromide, a carbamate prophylactic pretreatment drug for op nerve agent exposure. This publication cites certain recently published research on the effects of long term cognitive and/or neurophysiological effects of ops at low levels of exposure in animal models, e.g. Prendergast et al (1997) [where DFP was the test material] and in humans following acute exposures to sarin in a Tokyo train station, e.g. Murata et al (1997). This report of the Department of Veterans Affairs takes seriously the prospect that such low-level exposures can lead to long term cognitive, memory and other CNS effects that have not been adequately investigated. Accordingly, the stated purpose for future projects which the Department supports reads as follows: "This document presents a strategic approach to research on chemical warfare nerve agents that provides a framework for the organization, direction, and coordination of research on the long-term health effects from single and multiple organophosphate exposures to chemical warfare agents at doses ranging from the toxic, but non-lethal, down to the acute 'noobserved effect level' (NOEL). The framework encompasses the full spectrum of research from basic toxicology to epidemology, and is guided by accepted principles and paradigms for risk assessment." (p. 271) Also, evidently in reference to human studies: "Although many studies have been done to elucidate the acute effects of nerve agents, there is a paucity of studies on the long-term effects of these agents. A major reason for this is that people exposed to nerve agents, either accidentally or deliberately, had no apparent complaints in the months after the exposure to suggest the need for such studies." (p. 278)

Increasing levels of exposure result in progressively more serious effects, although the exact pattern of effects differs among anticholinesterase chemicals and may be influenced by the age of the patient***, (and much more). Since effects are progressive, how are the more subtle, but very important, cognitive effects that may occur in the range of minimal neural cholinesterase inhibition to be identified? (Absent adequate testing procedures for low dose effects, coupled with inadequate neural cholinesterase data, forces reliance upon the inhibition of blood enzymes for regulatory***

purposes.) Different cholinesterase-inhibiting chemicals may, and generally do, produce different spectra of clinical signs and behavioral effects. This complexity, in part, may arise from differences between absorbed chemicals in distribution between the central and peripheral nervous systems and differential binding in those nervous system compartments, or differential interactions with the two major types of cholinergic receptors (*i.e.*, muscarinic and nicotinic receptors). The nature and temporal pattern of effects also depends on the magnitude, duration, and frequency of exposure, as well as whether metabolic activation is needed. Perhaps one third of the effects caused by anticholinesterase chemicals (*e.g.*, headache, confusion, tremor, and convulsions) can be attributed primarily to effects on the central nervous system (Minton and Murray, 1988). For many effects, however, it is difficult to distinguish whether they are centrally or peripherally mediated or both.

OPP may require most or all of different kinds of toxicology studies in laboratory animals in support of the registration of a pesticide, including those which inhibit cholinesterase, depending upon the use/exposure pattern of the substance. Not all of these studies include the requirement for measurement of cholinesterase activity or the effects occurring as its consequence. The key studies are:

- Acute oral, dermal, and inhalation lethality tests in mammals;
- Acute or subchronic (90-day) delayed neurotoxicity study in hens;
- Acute or subchronic (90-day) neurotoxicity screening battery in rats, which includes:
  - ÿ Functional observational battery, which is a set of structured observations outside the home cage, including assessments of autonomic signs, pupillary response to light or pupil size, arousal, reactivity, posture and gait, grip strength, limb splay, and simple sensory reflexes (*e.g.*, tail pinch and a sudden sound);
  - ÿ Automated motor activity;
  - ÿ Histopathology of neural tissue from animals prepared by *in situ* perfusion;
  - ÿ Responses to visual or proprioceptive (i.e. sense of body position or awareness of pressure) stimuli are optional, but not commonly done.

    Should note the absence of assessments in this study of effects on such cognitive parameters as learning and memory, which even if subtle would be important where human endeavors are concerned.
  - 21-Day or subchronic (90-day) dermal toxicity study in mammals;
- Subchronic (90-day) inhalation study in mammals (if appropriate on basis of anticipated human route of exposure);
- Two chronic toxicity studies, one in the rat and one in the dog;
- Two prenatal developmental toxicity studies, one in a rodent and one in a non-rodent species; and
- Two-generation reproduction study in rodents; and

- Developmental neurotoxicity study in rats, which includes, in pups:
  - ÿ detailed observations, developmental landmarks, motor activity, auditory startle reflexes, learning and memory test, and neuropathology on postnatal days 11 and 60
  - ÿ detailed observations for neurological effects also are made in dams

A statement should be provided indicating which, if any, of these three studies incorporate cholinesterase assays, a critical end point in determining susceptability under FQPA.

While they never have been a part of EPA 's data requirements and, thus, there are no EPA testing guidelines for them, human hazard identification studies on some pesticides have been submitted by the chemical's sponsor(s) and, in the past, prior to the passage of FQPA, considered for use in risk assessments. These hazard identification studies typically are designed to identify no-effect levels for ChEI-associated enzyme activity and, sometimes, for some clinical effects. Although most of these human hazard identification studies with cholinesterase inhibitors are acute (i.e., single dose) in their exposure duration, a few have incorporated short-term (e.g., 4-10 day) or longer (e.g., 21-28 day) repeat dosing. Measures of cholinesterase inhibition in either whole blood (which is a mixture of plasma and RBCs), or separately in RBCs and plasma are usually included. Sometimes, reporting of some clinical symptoms and signs are included, e.g., in a few cases, objective physiological measures, such as blood pressure, have been reported.

Human hazard identification studies can be designed to detect more effects in addition to blood enzyme inhibition (*e.g.*, mild sweating and nausea) compared to animal studies, due to self-reporting of complaints, including sensory, cognitive, and psychological effects. Formal evaluations (by interview or test), however, are very uncommon as are measurements of physiological parameters like heart function (*e.g.*, heart rate and blood pressure) and breathing rate. More sophisticated neurobehavioral test batteries, such as intelligence tests or simple memory tests, used in epidemiological studies (for example, Anger *et al.*, 1996), are rarely, if ever, used in human hazard identification studies of cholinesterase-inhibiting organophosphorus and carbamate pesticides.

The reports of certain kinds of animal studies will include detections of overt clinical signs, including many of the autonomic signs, and motor effects, such as tremors. In the rodent neurotoxicity screening battery studies, the data are gathered systematically by observers unaware of treatment. The measurements of effects are defined quantitatively, albeit, usually on an ordinal scale (*e.g.*, +1, +2). Valid screening studies also include automated and quantitative measures of motor activity, grip strength, and limb splay, though changes in these measures are not a distinguishing characteristic of cholinesterase inhibition. EPA's test guidelines for the neurotoxicity screening batteries were published in 1991. OPP has received data from these neurotoxicity screening studies on many of the anticholinesterase organophosphorus and carbamate pesticides. Of the

roughly 30 effects that may occur following acute exposures as listed by Morgan (1989) and updated by Reigart and Roberts (1999), perhaps one third would not be seen in routine animal studies, or even in the neurotoxicity screening battery, as they are currently designed, especially the sensory, cognitive, and psychological effects. Thus, because of the limitations in the study design and conduct of both human and animal studies, OPP may not understand fully the profile of effects of concern that may result from exposure to the cholinesterase-inhibiting pesticides.

Conclusions:

- Inhibition of acetylcholinesterase in the nervous system (both central and peripheral) is generally accepted as a key component of the mechanism of toxicity leading to adverse cholinergic effects. The inhibition of this enzyme provides direct evidence of potential adverse effects. Interference with the timely deactivation of neuronal or neuroeffector acetylcholine results in the protraction of the actions of acetylcholine at these sites, which in turn results in adverse cholinergic effects. Because the inhibition of acetylcholinesterase is a key event that can lead to adverse effects, it provides valuable information in assessing potential hazards posed by anticholinesterase pesticides. ***The Background Document as presented to the SAP (1997), and affirmed by that Panel of experts, concluded that neural acetylcholinesterase inhibition satisfies those definitions of neurotoxicity cited in that review, and is thus to be regarded as a neurotoxic effect. (p. 20)***

- Measures of acetylcholinesterase activity in both central and peripheral nervous tissues are important for a full assessment of ***(actual and)*** potential hazard because the enzyme and each chemical may have different pharmacokinetic and pharmacodynamic properties in each compartment of the nervous system.

- The relationships between the functional effects and changes in acetylcholinesterase activity in both nervous system compartments is often difficult to characterize with existing data for a variety of reasons (*e.g.*, ***(development of tolerance)***, heterogeneity of cholinergic pathways including the molecular form(s) of AChE present at each location, limited data on the regional distribution of acetylcholinesterase, the time course of inhibition in each region, and limited evaluation of functional effects).

Rationale:

The inhibition of acetylcholinesterase is a key step in the mechanism of toxicity of certain organophosphorus and carbamate pesticides (Mileson, et al, 1998; ILSI 1999? Carbamate Report;

US EPA, 1999?? Carbamate paper for SAP), and, therefore, measures of cholinesterase inhibition represent a critical biochemical biomarker of potential adverse effects. ***As indicated previously, in accordance with definitions of neurotoxicity cited in the Background Document, neural acetylcholinesterase inhibition is a neurotoxic effect.*** Nonetheless, reductions in neural AChE activity may not always be accompanied by overt clinical signs or symptoms because, ***(for example, of the well documented phenomenon of tolerance as discussed in Dementi (1997), and supported by SAP (1997). SAP (1997) in fact says: "The development of tolerance during long-term exposures can 'mask' neurochemical changes induced by the anticholinesterases. Changes in receptor populations may therefore be able to explain discrepancies in studies wherein cholinesterase inhibition in target tissues does not appear to correlate with signs of toxicity...." (p. 23)),*** the critical functions of those specific neurons may not be sufficiently evaluated to detect related changes***, (and possibly other reasons such as flawed methodology.)*** The time at which potential functional effects are evaluated may also contribute to an apparent lack of concordance between functional effects and the neurochemical effects (*i.e.*, cholinesterase inhibition). Based on these limitations, it is difficult to determine, with accuracy or consistently, the degree of cholinesterase inhibition that will cause specific physiological or behavioral changes. ***[***Thus, OPP considers a treatment-related decrease in brain or peripheral tissue AChE activity, in itself, toxicologically important and data showing such a decrease appropriate for use as a critical effect for the derivation of RfDs and RfCs, as well as for characterizing potential human hazards.***] good***

Historically, data on central nervous system AChE inhibition have come from single or repeated exposure animal studies, in which whole brain homogenates are assayed at one or two time points. For the past five years, more detailed measurements of brain AChE inhibition have been required. These requirements, as part of the neurotoxicity screening battery, or as separate studies, have sought to characterize the time course of inhibition in plasma, RBCs, and brain, including in specific brain regions, after acute and 90-day exposures. Even so, most of the existing data sets will generally contain measures only of whole brain AChE activity, but not usually regional brain measurements, or time-course data, particularly following acute exposures. The lack of regional brain measures may be a limitation given that the distribution of cholinergic pathways and the concentration and molecular form of AChE in different brain regions is not homogenous. ***[***Thus, whole brain measurements of AChE inhibition may reveal little or no change in activity while masking significant

changes in specific brain regions associated with particular cholinergically-mediated functions (*e.g.*, the hippocampus and memory).***] Good. The very fact that neural acetylcholinesterase inhibition is assayed in so few compartments at so few time intervals, should serve to underscore the conclusion of the SAP (1997) (p. 20) to rely upon inhibition of the blood enzymes, plasma or erythrocyte, whichever is most sensitive, until such time as proper neural acetylcholinesterase data is obtainable and sensitive behavioral effects testing is performed.***

Unfortunately, measures of AChE inhibition in peripheral neural tissues or neuroeffector junctions are rare. AChE inhibition data from the peripheral nervous system have unique potential value because many of the adverse signs and symptoms associated with exposure to anticholinesterase pesticides (*e.g.*, diarrhea, excess salivation) are a result of effects on the peripheral nervous system. Because of the potential pharmacokinetic differences between the central and peripheral nervous compartments, measures of AChE activity in both of these systems are important for the full assessment of chemicals on the nervous system. Certain chemicals may have equivalent access to a specific compartment, in both degree and rate of interaction. On the other hand, there are many examples in which rate of access to, and concentration in, peripheral tissues is far greater than in the central nervous system. These patterns could shift with longer term exposures. ***The latter two sentences should be substantiated by a specific reference.***

Although AChE inhibition data in peripheral nervous system tissues have not been required in toxicological studies submitted to EPA and, at the moment, no standard protocol exists for the generation of such data, OPP indicated in 1997 that the collection of these measures could become a potential alternative to the use of blood cholinesterase inhibition measures in animal studies in the hazard and risk assessment process. As discussed earlier, the SAP (SAP, 1997) and an expert panel of ILSI (Mileson, et al. 1999) have stated that it is feasible to measure AChE inhibition in peripheral nervous system tissues. The 1997 SAP report asserted, "it is important that joint efforts be mounted to evaluate AChE inhibition in the peripheral neural tissues *per se* and in the neuroeffector junctions." The SAP expressed the view that it is technically feasible to routinely conduct AChE assays on the peripheral nervous system, while recognizing the difficulties involved. The SAP further suggested that skeletal muscles, heart, lung, salivary glands, diaphragm, and autonomic ganglia (*e.g.*, superior cervical ganglia) be considered as appropriate tissues to examine. The SAP considered that standardized and reproducible dissection and homogenization of tissue, assays with minimal tissue

dilution, selection of the most relevant tissue targets, and standardization of tissue storage conditions were the most important technical issues to resolve when measuring AChE activity in the peripheral nervous system. Work is underway in EPA's National Health and Environmental Effects Laboratory to develop and standardize protocols for assaying enzyme activity in various peripheral tissues (e.g. see Marshall, et al., 1999).

Conclusions:

• Inhibition of blood cholinesterases (*i.e.*, plasma and red blood cell) is not itself an adverse effect, but may indicate a potential for adverse effects on the nervous system. As a matter of science policy, blood cholinesterase data are considered appropriate surrogate measures of potential effects on peripheral nervous system acetylcholinesterase activity in animals***, (for CNS acetylcholinesterase inhibition in animals where CNS data is inadequate),*** and for both the peripheral and central nervous system acetylcholinesterase in humans.

• As such, blood cholinesterase inhibition data are considered appropriate endpoints for derivation of reference doses or concentrations when considered in a weight-of-the-evidence analysis of the entire database on a single pesticide or on two or more pesticides assigned to a common mechanism of toxicity group, where acetylcholinesterase inhibition is the common mechanism of toxicity.

• ***[***Red blood cell measures of acetylcholinesterase inhibition, if reliable, generally are preferred over plasma data***]***. ***The bracketed sentence is not consistent with the June 1997 Policy, nor the supporting Background Document, both of which were endorsed by the SAP (1997), and was not a conclusion of the SAP (1997). Therefore, it should be deleted and replaced with a more correct sentence, such as: (In the absence of adequate neural (CNS/PNS) acetylcholinestrerase inhibition data, inhibition of the blood enzymes, plasma or erythrocyte, whichever is most sensitive, should be employed as a surrogate for neural cholinesterase inhibition until such time as the question of relevance has been resolved by scientific means.) This approach is consistent with and supported by evidence presented in the Background Document, to which the reader is referred for the rationale, that among other reasons, plasma cholinesterase inhibition <u>often</u> correlates extremely well, and in a matter superior to that of erythrocyte acetylcholinesterase inhibition, with neural acetylcholinesterase inhibition; such that in general, until actual data has been obtained in each case illustrating one or the other of the blood enzymes as the superior neural cholinesterase surrogate, the presumption goes to the more sensitive responder of the blood enzymes. This is none other than a realistic and conscionable approach to be taken in the protection of the public health. In addition to all the rationale in the Background Document attesting to the importance to be assigned to plasma cholinesterase inhibition, I should note SAP***

(1997) says: "In a recent review of the California program, researchers found that plasma cholinesterase inhibition was predictive of pesticide-related illness." (p. 25) This is consonate with SAP's general recognition of both blood cholinesterases as important, with little distinction as to their relative importance as surrogates for neural cholinesterase inhibition and effects. In the interest of presenting a balanced and reliable assessment of the importance of plasma cholinesterase inhibition in the Policy statement, this should have been included. I should also note that the National Academy of Sciences, National Research Council, NRC (1993), concluded that children may be particularly vulnerable to organophosphate cholinesterase inhibitors in their diets, rationalized on the basis of a "common toxic effect", identified as plasma cholinesterase inhibition, resulting from the concerted effects of five such agents, namely: acephate, chlorpyrifos, dimethoate, disulfaton and ethion. (pp. 6, 297) In selecting this end point, NRC (1993) says: "This method was used to determine how many children are likely to be exposed to unsafe levels of multiple pesticides with that common effect....." (p. 297) Of course, one disturding outcome of this assessment based on the cholinesterase inhibition derived reference dose (RfD) was, as summarized in NRC (1993): "Through this new analytical procedure, the committee established that for some children, total organophosphate exposures may exceed the reference dose. Furthermore, although the data were weak, the committee estimated that for some children exposures could be sufficiently high to produce symptoms of acute organophosphate pesticide poisoning." (p. 7) Just as plasma cholinesterase inhibition is an expected response to a cholinesterase inhibitor, its inhibition has been used by experts (NRC) as a legitimate end point in addressing this public health issue. Since the red cell contains only acetylcholinesterase, the potential for exerting effects on neural or neuroeffector acetylcholinesterase may be better reflected by changes in red blood cell acetylcholinesterase than by changes in plasma cholinesterases which contain both butyrylcholinesterase and acetylcholinesterase in varying ratios depending upon the species. This conclusion rests on data showing that chemicals may have significantly differential affinities for binding with AChE and BuChE. ***There is no way that non quantitative based observations such as these can refute the fact that inhibitions of either of the blood enzymes may correlate better with inhibition of cholinesterase in one or more component(s) of the nervous system, given the numerous in vivo parameters that operate in determining relevance. Under any particular set of circumstances, relevance in question boils down to a game of chance more than anything that is predictible. Stated in its simplest terms, inhibition of either or both of the blood cholinesterase is the expected response resulting from exposure to a cholinesterase inhibiting pesticide, and should be taken seriously as evidence of neural cholinesterase inhibition in any one or more of its compartments or locations. Indeed, by chance alone, plasma cholinesterase inhibition may correlate better with one neural compartment, while erythrocyte cholinesterase inhibition may correlate better with the enzyme's inhibition in another compartment. Given the wide variety of molecular forms, differing activities in different neural compartments, differing recovery rates, etc., [Brimijoin (1992); Background Document] erythrocyte cholinesterase inhibition, at best, and by chance alone, can correlate with but one of the potentially many differing neural findings, and plasma inhibition likewise perhaps with another. Who is to say which is more important, but all are of concern, and the philosophy should be to rely upon the most sensitive in fulfilling our responsibilities to protect the public health. Now given what has been said here, it should be obvious (and this is supported by evidence cited in the Background Document), that inhibition of neither of the blood enzymes at times may be relied upon as evidence of neural cholinesterase inhibition. However, the latter may***

occur in the absence of measurable blood enzyme inhibition. This is why SAP (1997) says: ".....under SOME circumstances, measurments of SOME blood-borne cholinesterases would be appropriate to consider in establishing RfDs for anticholinesterases." (p. 21); and also says: "It was recognized that measured inhibition of cholinesterase activity in any of the blood fractions is best regarded as an imperfect mirror of enzyme inhibition in the true target tissues: brain, neuromuscular junctions, autonomic ganglia, and autonomic synapses." (p. 21) The point being, data on inhibition of cholinesterases in the blood, may or may not be conservative in the absence of representative neural data.

- Although RBC acetylcholinesterase data are generally preferred, in some cases, reliance on measures of RBC may not be appropriate because of methodological issues concerning blood measures of cholinesterase activity. When making weight-of-evidence judgments concerning the selection of RBC versus plasma measures of cholinesterase inhibition as endpoints for derivation of reference doses or concentrations, it is critical to consider all aspects of the information database, including the adequacy of the study protocol, quality of the data, dose-dependency of the responses, as well as available data on measures of brain acetylcholinesterase inhibition and functional effects.

- Plasma contains both butyrylcholinesterase and acetylcholinesterase in varying ratios depending upon the species. The separate characterization of RBC and plasma measures of cholinesterase inhibition provides some additional means of confirming results. Additionally, having separate RBC and plasma data allow for more informative animal-to-human comparisons.

- Work on standardizing methods for measuring acetycholinesterase activity in the peripheral nervous system is underway. OPP/EPA expects that the standardization and use of these methods will result in a database that improves the scientific understanding of the risks of cholinesterase-inhibiting compounds.

Rationale:

As a biomarker of exposure, blood cholinesterase inhibition is correlated with the extent of exposure. As discussed earlier, there is a direct relationship between a greater magnitude of exposure and an increase in incidence and severity of clinical signs and symptoms as well as blood cholinesterase inhibition. In other words, the greater the exposure, the greater the cholinesterase inhibition in the blood and the greater the potential for an adverse effect to occur. Both plasma and RBC measures of cholinesterase inhibition also provide:

ÿ pharmacokinetic evidence of absorption of the pesticide and/or its active metabolite(s) into the bloodstream and systemic circulation; and

ÿ pharmacodynamic evidence of binding to AChE, the neural form of the target enzyme, or to

plasma BuChE, an enzyme similar in structure to AChE

Because the interaction with AChE is widely accepted as a key event of the mechanism of toxicity for anticholinesterase pesticides, inhibition of this cholinesterase ***(or plasma cholinesterase)*** in the blood creates the assumption that a chemical also is causing inhibition of neural AChE. Chemicals are absorbed into the blood and transported to the peripheral nervous system. Pharmacokinetically, the blood compartment and the peripheral nervous system are outside the central nervous system ***(i.e., not accorded protection or shielded by the "blood-brain barrier", a regulatory interface.)*** Thus, blood measures of cholinesterase activity are viewed as a better surrogate for the effects on AChE in the peripheral nervous system than are enzyme changes in the central nervous system. Because data on AChE inhibition in the peripheral nervous system have rarely been gathered in animals, blood cholinesterase inhibition measures are generally the only information available to assess the potential of chemicals to inhibit AChE in the peripheral nervous system. In human studies, blood cholinesterase inhibition measures serve as surrogates for effects in both the central and peripheral nervous systems because neither of these neural tissues is available for evaluation directly. As discussed earlier, evaluations of clinical signs and symptoms have limitations, and thus should not be relied on solely, to the exclusion of other data. Therefore, blood cholinesterase inhibition data are considered appropriate endpoints for derivation of reference doses or concentrations when considered in a weight-of-the-evidence analysis of the entire database on a single pesticide or on two or more pesticides assigned to a common mechanism of toxicity group, where acetylcholinesterase inhibition is the common mechanism of toxicity.

The importance of blood cholinesterase inhibition data (RBC and plasma) is indicated by its use in monitoring workers for occupational exposures (even in the absence of signs, symptoms, or other behavioral effects). Blood cholinesterase inhibition (RBC and/or plasma) is considered as providing a sufficient basis for removing workers from the exposure environment. For example, the California Department of Health Services (CDHS) requires monitoring of agricultural workers who have contact with highly toxic organophosphorus or carbamate compounds (EPA Toxicity Category I or II pesticides; LD_{50} ÿ 500 mg/kg in rats)(CDHS, 1988). CDHS removes workers from the workplace whose plasma levels show 40% or greater cholinesterase inhibition from baseline, or whose red blood cell cholinesterase levels show 30% or greater inhibition. Workers may not be return until their cholinesterase values return to within 80% of baseline. The World Health

Organization (WHO) also has guidelines with the same RBC action levels (*i.e.*, 30% or greater inhibition), and considers plasma inhibition of 50% of baseline to indicate a "toxic" decrease (Fillmore and Lessinger, 1993). ***These practices attest to the relevance and importance of inhibitions of the blood enzymes (plasma cholinesterase included) as the expected responses following exposures to cholinesterase inhibitors. I have seen no evidence to justify a pesticide-wide differential use of plasma cholinesterase inhibition versus erythrocyte cholinesterase inhibition in the work place. It may have been worked out, as it should be, on a pesticide-by-pesticide basis, but as a generally applicable principle, I would question the practice. The author(s) of this Policy should not accept and use such claims, absent any citation to the data supporting them, in the context of the current debate over the relevance of the blood enzymes, a scientific issue. Certainly, one purpose of coming up with a Policy is to address the proper use of the agents in question. Its fine to say these people do this, but the grander question is whether they should be employing such principles. The Agency should take the lead in determining how these pesticide exposures should be controlled, after considering the scientific evidence, rather that citing the manner in which various organizations regulate exposures, as if that were some kind of scientific evidence.*** Fillmore and Lessinger also reviewed the California program and found that "The relative risk of pesticide poisoning was increased in workers whose initial baseline plasma levels were low, or if their levels had already dropped to 60-80% of their baseline previously in the season." ***In connection with this latter quotation, SAP (1997) had a little more to say: "In a recent review of the California program, researchers found that plasma cholinesterase inhibition was predictive of pesticide-related illness (emphasis added). They state this point as follows: 'The relative risk of pesticide poisoning was increased in workers whose initial baseline plasma levels were low, or if their levels had already dropped to 60-80 percent of their baseline previously in the season. (Fillmore C., Lessinger J.E. A cholinesterase testing program for pesticide applicators. Journal of Occupational Medicine, Volime 35, January 1993)" (p. 25)***

Although a pesticide's effect(s) on either RBC and plasma cholinesterase activity is considered to provide information on its potential to inhibit AChE in the nervous system, data from RBCs, which contain AChE exclusively, may better reflect neuronal AChE inhibition than data from the plasma, which is a variable mixture of butyrylcholinesterase and acetylcholinesterase. As discussed earlier, acetylcholinesterase is the enzyme involved in the mechanism of toxicity for the cholinergic effects

of anticholinesterase pesticides. Although BuChE is somewhat similar in structure to AChE, BuChE is nevertheless sufficiently different in important ways which often result in it having binding affinities to anticholinesterase agents that are quite different from those of acetylcholinesterase (Silver, 1974; Taylor and Radic, 1994) The composition of plasma cholinesterases varies widely among humans, dogs, and rats, the species for which these measures are most typically made. Human plasma is overwhelmingly BuChE with a ratio of BuChE to AChE of 1,000:1 (Edwards and Brimijoin, 1983). In dogs, there is a little more than 10% acetylcholinesterase in plasma with a ratio of BuChE to AChE of 7:1 (Scarsella *et al.*, 1979). In rats, plasma contains approximately 50% or more of AChE with a BuChE to AChE ratio of 1:3 in males and 2:1 in females (Edwards and Brimijoin, 1983). While it is technically possible to ascertain the contribution of each ChE to the level of inhibition in plasma, this type of data is rarely available. Thus, the relationship between blood measures of AChE and BuChE or other factors is usually not known. For these reasons, a treatment-related decrease in plasma cholinesterase activity, viewed in isolation, provides less insight into the potential of a chemical to cause neural AChE inhibition than do data on RBC AChE inhibition. ***Again, I question this assertion as not being in accord with the facts, excspt perhaps on an individual compound and circumstances of exposure basis.***

Historically, there have been technical difficulties with the measurement of the inhibition of plasma and RBC cholinesterase(s), particularly for the latter (see Wilson *et al.*, 1996). Although in recent years there have been improvements in blood measures of cholinesterase activity, it is important to consider carefully the methodological issues that may affect the accuracy and variability of the data when assessing the effects of pesticides on cholinesterase activity in blood. There are many methods available for measuring blood cholinesterase activity. The colorimetric method, based on the Ellman reaction, is considered a reliable method, and is commonly used for measuring plasma and RBC cholinesterase activity (Ellman, *et al.*, 1961; US EPA, 1992; ASCP, 1994). While well suited to the measurement of cholinesterase inhibition induced by organophosphorus pesticides, the Ellman method may underestimate cholinesterase activity in both plasma and RBC following carbamate exposure because of the relatively unstable binding of the carbamate esters to the acetylcholinesterase. The radiometric method may be better suited for measuring carbamate-inhibited cholinesterase (Johnson and Russell, 1975; Wilson, *et al.*, 1996). The refinement of measurement methods continues in NHEERL. ***The whole question of cholinesterase assay methodology is***

unresolved, and therefore causes me to have concern over the validity of in-house data.

The present science policy has been prepared considering the comments received from the SAP and the public in 1997 and during the public comment period in 1998. This revised policy continues to embrace the weight-of-the-evidence approach of considering all relevant data in an integrative manner that was described in the 1997 OPP document (US EPA, 1997). This revised policy expands the discussion of the approach and clarifies the weight-of-the-evidence approach by describing more explicitly under what conditions and how plasma and/or RBC cholinesterase data would be considered. ***The SAP (1997) in commenting on the weight-of-the-evidence approach says: "The question was deemed by the Panel to be of major importance. There was a consensus that the weight of the evidence approach is indeed reasonable and justified on the basis of the available scientific data so long as these data are derived from rigorous experiments with standardized methods and proper controls. In particular, this approach allowes flexibility to weigh heavily inhibition in non-target tissues when the overall toxicologic context suggests that other approaches pose danger of serious risk from overexposure." (p. 20). The reader should observe that, again, in referring to inhibition in non-target tissues, no distinction is rendered by Panel regarding the use of plasma as opposed to erythrocyte cholinesterase.*** The policy also re-emphasizes the potential usefulness of collection of peripheral neural data on AChE inhibition to reduce reliance on the surrogate blood measures.

OPP will use the weight-of-the-evidence approach described here to analyze individual studies as well as the complete database on a pesticide when selecting critical effects for hazard assessment. The primary objective of the weight-of-the-evidence analysis for anticholinesterase pesticides is to select Points of Departure (PoDs) (i.e., NOAELs, LOAELs, or benchmark doses) for critical effects to be used in the calculation of RfDs, RfCs or margins of exposure (MOE) for all of the routes and durations of exposure appropriate for a pesticide given its use and exposure patterns when, after review of the entire toxicological database, it is concluded that the cholinergic effect(s) induced by the substance being evaluated do, in fact, represent the critical effect(s). Briefly, the weight-of-the-evidence approach will include consideration of all available data on:

- clinical signs and other physiological and behavorial effects in humans and animals;
- symptoms in humans;
- central nervous system acetylcholinesterase inhibition;
- peripheral nervous system acetylcholinesterase inhibition;
- red blood cell acetylcholinesterase inhibition; and
- plasma cholinesterase inhibition (BuChE in humans; mixed AChE/BuChE in animals).

A comparison of the pattern of doses required to produce physiological and behavioral effects and cholinesterase inhibition in different compartments will be conducted. In addition to these parallel analyses of the dose-response information, comparisons of the temporal aspects (*e.g.*, time of onset and peak effects and duration of effects) of each relevant endpoint will be examined. This analysis should be done for each relevant route and duration of exposure (e.g., acute, intermediate and/or chronic exposures) for each available species/strain/sex of animals. Furthermore, the potential for differential sensitivity of adult versus young animals (*i.e.*, effects following perinatal or postnatal exposures) to anticholinesterase chemicals should be assessed. ***A statement should be included here explaining how cholinesterase data, the subject of this Policy, is to be used in addressing the differential sensitivity (susceptability) question as required under FQPA. It must be affirmed that the Guideline testing requirements for developmental toxicity, reproduction and developmental neurotoxicity studies, i.e. those studies relied upon most to satisfy the susceptability issue, do not incorporate cholinesterase assays. One would think that in consideration of the fact that inhibition of this enzyme is generally recognized as the most fundamental event on exposure to organophosphates and carbamates, and given that the blood-brain barrier may be poorly developed in young individuals, that assays of cholinesterase inhibition in young/developing individuals versus that in adults, would provide perhaps the most sensitive comparative data needed to make the call on susceptability. Clearly, the inclusion of cholinesterase assays in these studies should be called for in this Policy.*** These analyses should be conducted in the context of the adequacy of the protocols used and the quality of the available data. Based on this weight-of-the-evidence analysis for an anticholinesterase pesticide, OPP may select as the critical effects any one or more of the behavioral and physiological changes or enzyme measures listed above.

Although physiological and behavioral changes are considered very important for characterizing an adverse effect in humans, these endpoints are not given disproportionate emphasis or relied on solely, or even always necessarily preferred, in selecting critical effects for risk assessment because the evaluations of such endpoints have limitations. Comprehensive ***(emphasis added)*** measures of AChE inhibition in nervous system tissues***(, particularly in the young/developing individual versus the adult, as explained above)*** are considered important and are given considerable prominence in the weight-of-the-evidence analysis for selection of critical effects because, as discussed earlier, acetylcholinesterase inhibition is considered a key event in the mechanism of toxicity for the cholinesterase-inhibiting organophosphorus and carbamate pesticides and a substantial body of literature exists which links enzyme inhibition with a broad range of adverse effects. Thus, data on cholinesterase inhibition may be viewed as predictors of potential adverse responses mediated via cholinergic pathways and may be used instead of, or in the absence of, data on clinical signs and symptoms, and other physiological and behavioral effects. Direct measures of AChE inhibition in the neural target tissues, (*i.e.*, central and peripheral nervous systems) are preferred. However, when such data are missing or inadequate, they would obviously receive less weight in the analysis. In these circumstances, measures of cholinesterase inhibition in the blood (plasma and/or RBC) are viewed as reasonable surrogates for the peripheral nervous system given that the blood is the pharmacokinetic compartment into which chemicals are absorbed and transported to the peripheral nervous system. In animals, data on blood cholinesterase inhibition are also considered important companion data for central nervous system AChE inhibition, even though the brain constitutes a different pharmacokinetic compartment. As noted earlier, blood measures (both plasma and RBC) of cholinesterase activity in human studies must serve as surrogates for enzyme activity in both central and peripheral nervous systems, in light of the lack of availability of data on these parameters. As discussed in Chapter 3.3, within the blood compartment, RBC AChE data, if reliable, are generally preferred over plasma data. Even though plasma contains a mixture of AChE and BuChE, plasma cholinesterase data should be evaluated and considered in the parallel analyses as described below. As explained below, there may be certain situations where plasma cholinesterase inhibition may be selected as the critical effect for the risk assessment.

Evaluation of the statistical and toxicological significance of the study results and application of uncertainty factors will follow the Agency's established procedures for derivation of an RfD or RfC

and the principles articulated in the FQPA 10X Safety Factor policy. A description of the strengths, weaknesses, and limitations of the database will be included; this may lead to the identification of data needed to refine the data base and the risk assessment. Any residual concerns (i.e., significant uncertainties) will be accommodated when making the FQPA 10X Safety Factor determination.

Practically, the weight-of-the-evidence analysis may be viewed as having several steps: first, the individual studies are evaluated; second, all studies in the database and their relationship to one another are examined in an integrated manner; and lastly, the critical effects are selected for risk assessment and additional data needs identified. Below is a more detailed discussion of these steps.

For a full evaluation of an anticholinesterase pesticide, the important elements of a study should include:

•Evaluations of physiological and behavioral effects;

•Measures of central nervous system acetylcholinesterase activity in animals (often these will be whole brain measures rather than measures in specific brain regions) ***(Given the wide ranging cholinesterase inhibition and rates of recovery in the various brain regions, Brimijoin (1992), in this case more reliance will need to be placed on cholinesterase inhibition in the non-target tissues, according to SAP (1997). (p. 20))***

•Measures of peripheral nervous system acetylcholinesterase inhibition in animals (rarely available at the present time);

•Measures of RBC and plasma cholinesterase inhibition.

First, each study is critically evaluated. This evaluation involves consideration of, among other factors: the adequacy of study protocol and design (*e.g.*, treatment group size, dose spacing, methods used for neurochemical and functional evaluations), whether pre-exposure data were obtained, and the conduct of the study. Results should be assessed in the context of both statistical and biological significance. The consistency of the findings within the study when repeated measures are taken, the dose-dependency of the responses, as well as the temporal aspects of effects (*e.g.*, the time-of-onset, steady state, time-to-peak effects and the time until complete recovery) are to be examined. The relationship of the different effects seen to one another should also be considered in interpreting the findings. Following critical evaluation of the validity of the study, candidate points of departure (i.e. NOAELs, LOAELs, or benchmark

doses) are identified or calculated.

4.2 INTEGRATIVE ANALYSIS OF THE DATA BASE

When evaluating the entire database and selecting an endpoint(s) as the critical effect(s) to serve as the PoD in the derivation of a RfD or RfC, parallel analyses of the dose-response (i.e., changes in magnitude of enzyme inhibition or of a different effect with increasing dose) and the temporal pattern of all relevant effects will be compared across all of the different compartments affected (*e.g*., plasma, RBC, peripheral nervous system, brain), and for the functional changes to the extent the data base permits. The overall adequacy of the test protocols and the quality of the data also will be important elements of the analysis. The consistency of LOAELs, NOAELs, or BMDs for each category of effects (*e.g.*, clinical signs, ***As stated earlier, classical cholinergic clinical signs/symptoms do not address the question of the possible presence of 1) more subtle but very important cognitive or behavioral effects that could be identified by specific testing procedures, or challenges; 2) tolerance; 3) remarkable changes in neurochemistry.*** cholinesterase inhibition in the various compartments, etc.) for the test species/strains/sex available and for each duration and route of exposure should be noted. If scientifically valid, reliable, and ethically appropriate to use, human data may be preferable to animal data because they preclude the need for extrapolation of results across species, avoiding the uncertainties attendant to this aspect of the risk assessment process. Confidence in the selection of an endpoint(s) for derivation of an RfD or RfC will be enhanced by the factors described in Table 1. The findings for anticholinesterase pesticides will span a broad continuum, and their databases will range from those which are comprehensive and robust to those which are limited and of poor quality. Thus, end point selection and weight-of-evidence judgments must be made on a case-by-case basis. For example, often cholinesterase inhibition data in a single compartment may be inconsistent across studies involving the same species or strain. In some cases, large differences may be noted in the magnitude of cholinesterase inhibition in one compartment in comparison to all the other compartments. In other cases, there is no dose-effect relationship for cholinesterase inhibition in one or more compartments. Time course data for cholinesterase inhibition also are often limited. Brain measures of AChE activity are often limited to whole brain at termination of an animal study. So a typical database for an anticholinesterase pesticide will likely contain a number of inadequacies that can have a broad spectrum of influence from none to substantial on the selection of critical effects. It should be emphasized, however, that the lack of, or deficiency in, any one factor listed in Table 1 would not <u>necessarily</u>***, emphasis added,*** discount the usefulness of a study in selecting an endpoint for calculation of an RfD or RfC. ***How can this statement hold true if the***

most sensitive or critical end point in the study, the one that would indeed toe the line is missing or compromised? For example, suppose the most critical end point is cholinesterase inhibition in a subchronic data set, but cholinesterase data are inadequate because the study requires too few animals to yield reliable assessments of cholinesterase inhibition, i.e. the statistics are weak. I assume its implicit that study deficiencies would have to be satisfied. On the other hand, suppose it were cholinesterase data in a reproduction study, an end point not required to be assayed?

Functional evaluations are limited in both human and animal studies. Therefore, as described earlier, measures of cholinesterase inhibition are included in the weight-of-the-evidence evaluation; the reliance on all relevant data is considered to be both scientifically sound and public health protective. Cholinesterase inhibition in the blood may occur at lower doses than other cholinergic effects (*e.g.*, brain AChE inhibition, functional effects). ***Blood cholinesterase inhibition cannot always be relied upon to yield the most sensitive response to cholinesterase inhibitors. Effects in the CNS may be more remarkable, Background Document (p. 19)*** The NOAEL or equivalent benchmark dose for RBC AChE inhibition and that for plasma and/or brain may not be the same. This could be due to methodological problems or to the different binding affinities of a pesticide to AChE compared to those for BuChE or to a number of other factors. As explained in Section 3.3, if the measurements of AChE inhibition in RBCs are considered methodologically sound, these data generally are preferred over plasma cholinesterase activity data as predictors of neural AChE activity, even if the plasma NOAEL/BMD is lower. ***As stated before, not unless it has been determined to be true.*** However, if the RBC data are unreliable (*e.g.*, questions exist about the methodology or there is no dose-dependency) or the dose response for inhibition of plasma cholinesterase more closely approximates that for AChE inhibition in the nervous system than does the dose response for RBC acetylcholinesterase inhibition, plasma cholinesterase inhibition may be the more prudent endpoint to use to represent the critical effect. Occasionally, because of methodological difficulties or for other, poorly-understood reasons, empirical correlations between the doses that cause plasma and brain cholinesterase inhibition (in the same or other studies) may be stronger than those between the doses for RBC and brain enzyme inhibition. ***Also, given this acknowledgment, doesn't it instruct that the determination of relevance must be required for regulatory purposes, lest the more sensitive responder be employed?***

The weight-of-the-evidence approach emphasizes the determination of the quality of the cholinesterase data, especially the RBC measures. ***No more so than for plasma.*** Standard operating procedures for measuring cholinesterase activity have continued to evolve over the last decade (Wilson *et. al.,* 1996; Hunter *et. al.,* 1999); detailed information on the method(s) and procedures used for measurements of cholinesterase activity following treatment is important. The method used for carbamate pesticides is particularly important because the reliability of data on cholinesterase effects depends not only on the specific methodology used, but to a great extent on sample processing (given the readily reversible nature of the carbamylated AChE).

As discussed earlier, the 1997 FIFRA Scientific Advisory Panel endorsed collection of peripheral nervous system AChE data as being technically feasible and advised OPP that these data may be a better indicator of cholinergic effects than blood cholinesterase measures. The ILSI Panel provided more technical guidance along with a number of recommendations for further studies to improve the methodologies, while, nonetheless concluding that such measures could be taken now (Mileson, et al., 1999). OPP agrees that peripheral nervous system measurements from a suitable set of tissues could provide an alternative to blood measures. OPP, with ongoing technical and research support from NHEERL, will continue to support the development and validation of methodologies for measuring peripheral neural AChE inhibition. This was a major aspect of OPP's policy in 1997 and was endorsed by the 1997 SAP. Once a methodology is validated, data from such studies will be sought on a regular basis and used to supplement or replace blood measures which now serve as a surrogate for the peripheral nervous system. In the interim, any data on peripheral tissues will be evaluated and incorporated into the risk assessment on a case-by-case basis. OPP strongly encourages the development of any data aimed at refining risk assessments based upon blood measures to be focused on peripheral nervous system measures of AChE. Additional data to differentiate between the acetylcholinesterase and butyrylcholinesterase in plasma, a procedure recommended by the SAP in 1997, could also be useful.

Additional studies to provide data on metabolism, pharmacokinetics and pharmacodynamics also may be useful to aid in the characterization of the cholinesterase inhibiting properties and

potential hazard of organophophorus and carbamate pesticides. To lessen the uncertainties inherent in route-to-route extrapolation, endpoint specific data could be collected on exposure routes of interest, such as cholinesterase inhibition following dermal exposure.

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The elements of the weight-of-the-evidence evaluations used for selecting toxicity endpoint(s) are summarized in Table 2. Weight-of-the-evidence judgments must be sound and supported by the data on the individual pesticides. The risk assessor should provide a hazard characterization that summarizes the endpoint data that were available for consideration, discusses the strengths, limitations and uncertainties of the data, and describes how well the data supports the conclusions. The rationale for selection of the critical effect(s) must be clearly articulated in this characterization.

ASCP.

6. REFERENCES

ASCP 1994. American Society of Clinical Pathologists. Check Sample: Cholinesterase: Review of methods. Clinical Chemistry 34 (10):135-157.

Anger WK, Otto DA, Letz R. 1996. Symposium on computerized behavioral testing of humans in neurotoxicology research: Overview of proceedings. Neurotox. Teratol. 18(4): 347-350.

Brimijoin, S. 1992. Enzymology and Biology of Cholinesterases. In: Proceedings of the US EPA Workshop on Cholinesterase Methodologies. US EPA, 1992. Washington, DC

CDHS. 1988. California Department of Health Services. Medical Supervision of Pesticide Workers: Guidelines for Physicians.

CDPR. 1997.California Environmental Protection Agency-Department of Pesticide Regulation. Use of Cholinesterase Inhibition Data in Risk Assessments for Pesticides. May 16, 1997. 8 pp.

Dementi, B. 1997. Cholinesterase Literature Review and Comment, Part*s A and* B. FIFRA Scientific Advisory Panel Docket OPP #0048, May 1, 1997.

Ecobichon, D.J. 1996. Toxic effects of pesticides. In Unit 5, Toxic Agents of: Casarett & Doull's Toxicology: The Basic Science of Poisons (C.D. Klaassen, M.O. Amdur, and J. Doull, eds.) McGraw-Hill, New York. pp. 643-689.

Edwards J.A. and Brimijoin S. 1983. Effects on hypophysectomy on acetylcholinesterase and butyrylcholinesterase in the rat. Biochem. Pharmacol. 32 :1183-1189.

Ellman, G.L., et al. 1961. A new and rapid colormetric determination of acetylcholinesterase activity. Biochem. Pharmacol 7:88-95.

Fillmore CM and Lessinger JE. 1993. A Cholinesterase Testing Program for Pesticide Applicators. J Occup. Med. 35 (1): 61-70.

Hoffman BB, Lefkowitz RJ, Taylor P. 1996. Chapter 6-Neurotransmission: The Autonomic and Somatic Motor Nervous Systems. In: The Pharmacological Basis of Therapeutics, 9th Edition, (Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Gilman AG, Eds.) McGraw-Hill, New York, pp. 105-139.

Hunter DL, Marshall RS, Padilla S. 1997. Automated Instrument Analysis of Cholinesterase Activity in Tissues From Carbamate-Treated Animals: A Cautionary Note. Toxicol. Methods 7(1):43-53.

ILSI. Report on Carbamates Mechanism of Action

JMPR. 1999. FAO/WHO Joint Meeting on Pesticide Residues. Report of the 1998 FAO/WHO Joint Meeting on Pesticide Residues. Food and Agricultural Organization-United Nations. Rome, Italy. p.??????

Johnson, C.D. and Russell, R.L. 1975. A rapid, simple radiometric assay for cholinesterase suitable for multiple determinations. Analytical Biochem. 64: 229-238.

Marshall RS, Lassiter TL, Padilla S. 1999. A Method for Specifically Assaying Acetylcholinesterase Activity in Peripheral Tissues. Toxicol. Sciences. 48 (1):374.

Mileson BE, Chambers JE, Chen WL, Dettbarn W, Ehrich M, Eldefrawi AT, Gaylor DW, Hamernik K, Hodgson EH, Karczmar AG, Padilla S, Pope CN, Richardson RJ, Saunders DR, Sheets LP, Sultatos LG, Wallace KB. 1998. Common Mechanism of Toxicity: A case study of organophosphorus pesticides. Toxicol. Sciences 41: 8-20.

Mileson BE, Brimijoin S, Chambers JE, Dass PD, Padilla S, Sheets LP, Taylor PW, Van Pelt C, Wallace KB. 1999. Guidance for the Design and Interpretation of Studies Intended to Characterize Acetylcholinesterase Activity in the Peripheral Nervous System. 47pp. (Unpublished Report of the International Life Sciences Institute, Risk Sciences Institute, Washington, DC).

Minton NA and Murray VSG. 1988. A review of organophosphate poisoning. Med. Toxicol. 3: 350-375.

Morgan, DP 1989. Recognition and Management of Pesticide Poisonings, 4th Edition. US EPA (US Environmental Protection Agency, Washington, DC EPA 540/9-88-001.

NRC/NAS. 1983. National Research Council/National Academy of Sciences. Risk Assessment in

the Federal Government: Managing the Process. National Academy Press, Washington DC.

NRC/NAS. 1994. National Research Council/National Academy of Sciences. Science and Judgment in Risk Assessment. National Academy Press, Washington, DC.

PMRA-Canada. 1997. Pesticide Management Regulatory Agency-Canada. Their CheI Policy.

Scarsella, G, Toschi G, Bareggi SR, Giacobini E. 1979. Molecular forms of cholinesterase in cerebrospinal fluid, blood plasma, and brain tissue of the beagle dog. J Neurosci. Res 4:19-24.

Reigart, R. and J. Roberts. Recognition and Management of Pesticide Poisonings, 5th Edition. US EPA (US Environmental Protection Agency, Washington, DC EPA 735-R-98-003

SAB/SAP. 1990. Science Advisory Board/Scientific Advisory Panel. Report of the SAB/SAP Joint Study Group on Cholinesterase. Review of Cholinesterase Inhibition and its Effects. EPA-SAB-EC-90-014. 17 pp.

SAB/SAP. 1992. FIFRA Scientific Advisory Panel. Report of the SAB/SAP Meeting 11/6/92. A Set of Scientific Issues Being Considered by the Agency in Connection with Aldicarb and Aldicarb Sulfone. 11/25/92.

SAB/SAP 1993. Science Advisory Broad/Scientific Advisory Panel. An SAB Report: Cholinesterase Inhibition and Risk Assessment. Review of the Risk Assessment Forum's Draft Guidance on the Use of Data on Cholinesterase Inhibition in Risk Assessment by the Joint SAB/SAP Joint Committee. EPA-SAB-EHC-93-011. 20 pp.

SAP. 1997. FIFRA Scientific Advisory Panel. Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel Meeting Report. A Set of Scientific Issues Being Considered by the Agency Concerning the Office of Pesticide Programs (OPP)-Cholinesterase Inhibition Policy. July 1997. pp.18-25.

Silver A. 1974. Biology of Cholinesterases. North Holland Publishing Company, Amsterdam, The Netherlands. 596 pp.

Taylor P. 1996a. Chapter 8: Anticholinesterase Agents. In: The Pharmacological Basis of Therapeutics, 9th Edition, (Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Goodman Gilman A, Eds.) McGraw-Hill, New York. pp. 161-176.

Taylor P. 1996b. Chapter 9: Agents Acting at the Neuromuscular Junction and Autonomic Ganglia. In: The Pharmacological Basis of Therapeutics, 9th Edition, (Hardman JG, Limbird LE, Molinoff PB, Ruddon RW, Goodman Gilman A, Eds.) McGraw-Hill, New York. pp. 177-198.

Taylor P, Radic Z. 1994. The cholinesterases: from genes to proteins. Annual. Rev. Pharmacol. Toxicol. 34:281-320.

US EPA. 1988. US Environmental Protection Agency. Risk Assessment Forum. Discussion Summary of the Colloquium on Cholinesterase Inhibition. June30, 1988.

US EPA. 1992. US Environmental Protection Agency. Proceedings of the U.S. EPA Workshop on Cholinesterase Methodologies. February 19, 1992.

US EPA. 1994. US Environmental Protection Agency. Methods for derivation of inhalation reference concentrations and application of inhalation dosimetry. EPA/600/8-90/066F.

US EPA. 1995. US Environmental Protection Agency. The Use of the Benchmark Dose Approach in Health Risk Assessment. EPA/630/R-94/007.

US EPA. 1996. US Environmental Protection Agency. Benchmark Dose Technical Guidance Document, EPA/600/P-96/002A.

US EPA. 1997a. .US Environmental Protection Agency. Office of Pesticide Programs Reference Dose Tracking Report (3/28/97)

US EPA. 1997b. US Environmental Protection Agency). Federal Insecticide, Fungicide, and Rodenticide Act Scientific Advisory Panel Open Meeting, June 4, 1997. A Set of Scientific Issues Being Considered by the Agency Concerning the Office of Pesticide Programs (OPP) Cholinesterase Inhibition Policy. Docket OPP#00480.

US EPA. 1998a. US Environmental Protection Agency. Guidelines for Neurotoxicity Risk Assessment. Federal Register 93:26926-26954. 14 May 1998.

US EPA. 1998b. US Environmental Protection Agency. Science Policy Issues Related to the Food Quality Protection Act. Office of Pesticide Program's Science Policy on the use of Cholinesterase Inhibition for Risk Assessments of Organophosphate and Carbamate Pesticides, OPP Docket #00560. Federal Register 63(214):59780-59783. 11/5/98.

US EPA. 1998c. US Environmental Protection Agency. Science Advisory Board/Scientific Advisory Panel; Notification of Public Advisory Committee Meeting. Federal Register 63(225): 64714-64715.

US EPA. 1999a. US Environmental Protection Agency. Policy on a Common Mechanism of Action: The Organophosphate Pesticides. THIS PAPER IS NOT YET AVAILABLE ON THE INTERNET

US EPA. 1999???. US Environmental Protection Agency. Science Advisory Board/Scientific Advisory Panel; Notification of Public Advisory Committee Meeting. Federal Register 64 (205): 57452-57455. October 25.

US EPA. 1999. US Environmental Protection Agency. The Office of Pesticide Programs' Policy on Determination of the Appropriate FQPA Safety Factor(s) for Use in the Tolerance-setting Process. Draft proposal. Federal Register 64 (130): 37001-37009. July 8.

US EPA. 1999???. US Environmental Protection Agency. The Carbamate Pesticides and the Groupoing of Carbamates with the Organophosphorus Pesticides. Presented to the FIFRA Scientific Advisory Panel September, 1999.

Wilson BW, Padilla S, Henderson JD, Brimijoin S, Dass PD, Elliot G, Jaeger B, Lanz D, Pearson R, Spies R. 1996. Factors in Standardizing Automated Cholinesterase Assays. J Toxicol. Env. Health 48:187-195.

s. uce, or adapt to the environment. Neurotoxicity is defined as an adverse change in the structure or function of the central and/or peripheral nervous system following exposure to an agent (US EPA 1998a).

t. (IRIS) as "the first adverse effect, or its known precursor, that occurs as the dose rate increases." Information on the derivation of reference doses or reference concentrations can be found at the IRIS website http://www.epa.gov/ngispgm3/iris/rfd.htm.

.. k software for hazard endpoints is currently available at http:\www.epa.gov\ncea\bmds.

... toxicity of anticholinesterase chemicals can be found in several chapters of two widely-available textbooks (Ecobichon (1996), Hoffman, et al., (1996), and Taylor (1996a, 1996b)) and in Dementi (1997) which served as a technical support document to the 1997 policy document.

4.

s. d as a condition which is a departure from normal function reported by the person experiencing and reporting that condition (*e.g.*, headache, nausea). A clinical sign is an objectively-measured effect (*e.g*., heart rate, blood pressure) indicative or suggestive of a condition for an individual (or animal) observed and recorded by another such as a physician.

ss. its policy concerning human studies with respect to ethical and scientific standards for their acceptability and use in risk assessments, particularly with respect to decisions under FQPA. The Agency neither requires nor encourages the conduct of human hazard identification studies to detect potential adverse effects of pesticides. EPA has held two meetings of a joint SAP/SAB (Science Advisory Board) panel (December 1998 and November, 1999 (US EPA, 1998c,1999)) on the ethical elements of this issue. The Panel's report is expected in the Spring of 2000.